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The effect of down-regulation of mitochondrial RNA-binding proteins MRP1 and MRP2 on respiratory complexes in procyclic *Trypanosoma brucei*

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Abstract

MRP1 and MRP2 are multifunctional *m*itochondrial *R*NA-binding *p*roteins with a regulatory role in RNA editing and putative role(s) in RNA processing in *Trypanosoma brucei*. Silencing of MRP1 and/or MRP2 by RNA interference affected the assembly and functionality of respiratory complexes. The absence of several subunits of complexes I, III and IV resulted in their disintegration and subsequent decrease of specific activities and also caused a significant decrease of membrane potential. The overall respiration in the interfered cells decreased by only about 20%, since the trypanosome alternative oxidase effectively replaced the missing cytochromes and became the principal terminal oxidase.

Keywords: Mitochondrial respiratory chain; Alternative oxidase; Trypanosome; RNA interference; RNA editing; RNA-binding proteins

1. Introduction

Trypanosoma brucei and related kinetoplastid flagellates kill hundreds of thousands of people annually, and debilitate millions. Current therapy for African sleeping sickness, leishmaniases and Chagas disease is inefficient and is based on decades old drugs that have severe side effects. The development of novel drugs directed against targets that are unique to the parasite is thus highly desirable. RNA editing of mitochondrial transcripts belongs to promising targets, since several proteins that participate in this pathway have no sequence similarity with proteins of the human host.

In the mitochondrion of *T. brucei* editing is responsible for (extensive) post-transcriptional modification of a subset of mRNAs. Without editing, these transcripts contain multiple frameshifts and/or lack start and stop codons and cannot therefore be translated into functional proteins. The requisite genetic information for editing is provided by small non-catalytic guide (g) RNA molecules. Proteins responsible for the enzymatic cascade leading to proper insertion/deletion of uridines at specific sites constitute a large complex termed editosome [1–3]. Moreover, several proteins that are not stable part of the editosome appear to be essential for editing, since their down-regulation or absence causes disruption of the editing process. Prominent members of this group are MRP1 and MRP2, abundant *m*itochondrial *R*NA-binding *p*roteins that have been cross-linked to gRNAs and associate in a heterotetrameric complex. MRPs have a mutual dependence for stability, as the loss of one MRP leads to the loss of the other [4] and their interaction with the editosome is likely mediated through the RNA molecules [4–6]. Moreover, MRP1, which binds gRNAs with nM affinity, was shown to have matchmaking activity in vitro [7], and is though to promote the annealing of the gRNA with cognate mRNA by neutralizing the negative charge of the bound gRNA [7,8].

Down-regulation of MRP1, MRP2 or both by RNA interference (RNAi) affected editing and/or stability of several mitochondrial mRNAs. As shown by poisoned primer extension, in

Abbreviations: TAO, trypanosome alternative oxidase; apoC, apocytochrome c_1; apoB, apocytochrome B; cox, cytochrome *c* oxidase; MRP, mitochondrial RNA-binding protein; hsp70, heat-shock protein 70; trCOIV, trypanosome cytochrome *c* oxidase subunit IV; 2D, two-dimensional

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all knock-down cell lines, at least one mRNA encoding a presumably essential subunit of the respiratory complexes I, III and IV was either destabilized or its editing was compromised. On the other hand, the only mitochondrial-encoded subunit of the respiratory complex V, the mRNA of which undergoes pan-editing, was unaffected by the depletion of the MRP proteins [4] (also see Section 4). While phenotypes caused by the disruption or down-regulation of many editing-associated proteins have been described in detail on the level of RNA [3], almost nothing is known about the consequences at the level of mitochondrial function and biogenesis. In fact, de novo protein synthesis and the stability of respiratory complexes have only been studied in cells in which terminal uridylyl transferase I, a key protein in the *T. brucei* RNA editing, was silenced [9]. Herein, we have analyzed the pleiotrophic effect of RNAi down-regulation of MRP1 and/or MRP2 on the stability and function of respiratory complexes, as well as the respiration and mitochondrial membrane potential. We show that the decreased abundance of a subset of edited and never-edited transcripts has a dramatic effect on several key mitochondrial functions.

2. Materials and methods

2.1. Trypanosome culture, RNAi induction and growth

The transgenic procyclic cell line (29-13 parental strain) was cultured in SDM-79 medium with 10% foetal calf serum at 27 °C. The cell lines stably transfected with the MRP1 and/or MRP2 expression constructs have been described previously [4]. RNAi was induced with 1 μg/ml tetracycline. Induced cells were maintained in the log-phase growth by daily dilutions and harvested at day 6 after RNAi induction.

2.2. Western blot analysis

Cell lysates of the *T. brucei* parental strain, and non-induced and induced cell lines were loaded on a 12% SDS-PAGE gel (5×10^6 cells per lane), blotted and probed with primary antibodies. The polyclonal rabbit antibodies against the *T. brucei* apocytochrome c_1 (apoC) (1:500) (provided by S.L. Hajduk), ATPase subunit b (1:1000) (provided by L. Simpson), cytochrome *c* oxidase subunit 6 (cox6) (1:500), mitochondrial RNA-binding protein 1 (MRP1) (1:1000) and 2 (MRP2) (1:1000), monoclonal mouse antibodies against the *T. brucei* heat-shock protein 70 (hsp70) (1:2000) (provided by K. Stuart), trypanosome alternative oxidase (TAO) (1:100) (provided by G.C. Hill) and *Leishmania tarentolae* cytochrome *c* oxidase subunit IV (trCOIV) (1:500) were used at dilutions shown in brackets. Secondary anti-rabbit and anti-mouse IgG antibodies (1:1000) (Sevapharma) coupled to horseradish peroxidase were visualized according to the manufacturer's protocol using the ECL kit (Amersham Biosciences).

2.3. Isolation of kinetoplast–mitochondrial vesicles

The kinetoplast–mitochondrial vesicles from 5×10^8 cells were isolated by hypotonic lysis as described elsewhere [10]. Pelleted mitochondrial vesicles were stored at −70 °C.

2.4. Two-dimensional gel electrophoresis

Analysis of the respiratory complexes from purified mitochondria was performed by two-dimensional (2D) blue native/Tricine–SDS-PAGE gels. Mitochondria isolated from 5×10^8 cells were resuspended in 40 μl of 1 M aminocaproic acid (ACA) and the addition of 10 μl of 10% dodecylmaltoside was followed by 1 h incubation on ice. The lysate was spun in a microcentrifuge (12,000 rpm, 10 min, 4 °C) and protein concentration was determined by the Bradford assay. One hundred micrograms of total protein was mixed with 1 M ACA and 1.5 μl of CB solution (0.5 M ACA and 5% [w/v] Coomassie Brilliant Blue G-250) to final volume 20 μl. The mixture was incubated for 10 min on ice and loaded on a 2–15% gradient blue native gel followed by resolution in a 10% Tricine–SDS-PAGE gel. After electrophoresis the gels were stained with Coomassie Brilliant Blue R250.

2.5. NADH dehydrogenase, cytochrome c reductase and oxidase activity assays

NADH dehydrogenase (complex I) activity was measured in a 1 ml cuvette containing the NDH buffer (50 mM potassium phosphate buffer, pH 7.5; 1 mM EDTA; 0.2 mM KCN), 5 μl of mitochondrial lysate and 5 μl of 20 mM NADH. After the addition of 10 μl of a mixture of 2 mM coenzyme Q_2 and 5 mM ferricyanide, the change in absorbance at 340 nm was measured every 10 s for 3 min.

Cytochrome *c* reductase (complex III) activity was measured in a 1 ml cuvette containing the QCR buffer (40 mM sodium phosphate buffer, pH 7.4; 0.5 mM EDTA; 20 mM sodium malonate; 50 μM horse heart cytochrome *c* [Sigma]; 0.005% dodecylmaltoside). After the parallel addition of 2 μl of mitochondrial lysate and 2 μl of 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinol (DMB; Sigma), the solution was quickly mixed with a pipette, and the change in absorbance at 550 nm was measured every 5 s over a period of 60 s. DMB was initially reduced as described elsewhere [10]. Potassium cyanide (KCN) was added to the final concentration 200 μM as an inhibitor of the interfering oxidase activity.

Cytochrome *c* oxidase (complex IV) activity was measured in a 1 ml cuvette containing the COX buffer (40 mM sodium phosphate buffer, pH 7.4; 0.5 mM EDTA; 30 μM sodium ascorbate; 20 μM horse heart cytochrome *c* [Sigma]; 0.005% dodecylmaltoside) to which 10 μl of mitochondrial lysate was added. The change in absorbance at 550 nm was measured every 20 s over a period of 10 min. Antimycin was added to the final concentration of 300 ng ml^{-1} as an inhibitor of the interfering reductase activity.

For complex I, a unit of activity was defined as an amount of enzyme that catalyses the oxidation of 1 nmol of NADH per min, assuming an extinction coefficient of 6.2 mM^{-1} cm^{-1} [11]. For complexes III and IV, a unit of activity was defined as the amount of enzyme that catalyses the reduction or oxidation of 1 μmol of cytochrome *c* per min, assuming an extinction coefficient of 21.1 mM^{-1} cm^{-1} [12].

2.6. Histochemical staining of complexes I and IV

For the staining of complex I, 30–130 μg of mitochondrial lysate, prepared as described above, was loaded per lane and run on a 2–15% gradient blue native PAGE gel. Immediately after the run, the gel was stained in the reaction buffer A (0.1 M Tris–HCl, pH 7.4; 0.14 mM NADH, 1 mg/ml nitrotetrazolium blue chloride) by slow agitation overnight. For the staining of complex IV, the mitochondrial lysate was resolved under the same conditions and after the run, the gel was stained in the reaction buffer B (50 mM phosphate buffer, pH 7.4; 1 mg/ml 3,3′-diaminobenzidine; 24 U/ml catalase; 1 mg/ml cytochrome *c*; 75 mg/ml sucrose) by slow agitation overnight. The enzymatic activities of complexes I and IV appear as specific colored precipitates (violet for complex I and brown for complex IV). The gels were subsequently fixed in a mixture of 30% methanol and 10% acetic acid [13]. Destained gels were examined by Kodak Image Station IS 4000 mm and the precipitates were quantified using Kodak Molecular Imaging System.

2.7. Measurement of respiration rate and membrane potential

Logarithmically growing cells were collected, washed and resuspended in 1 ml of SDM-79 medium at a concentration of 3×10^7 cells/ml. Oxygen consumption was determined with a Clark-type polarographic electrode (1302 Microcathode Oxygen Electrode, model 782; Strathkelvin Instruments). KCN and salicylhydroxamic acid (SHAM) were added in 4 min intervals to final concentrations of 0.1 and 0.03 mM, respectively.

Tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) uptake was used to measure the mitochondrial membrane potential. 1×10^6 cells were incubated in 1 ml of SDM-79 medium with 0.4% TMRE for 20 min at 27 °C. After staining, the cells were pelleted by centrifugation at 2500 rpm for 5 min, resuspended in a dye-free medium and immediately used for flow cytometry analysis, with 20,000 events measured in each experiment using an Epics XL flow cytometer (Coulter). The data were analyzed using WinMDI software.

3. Results

3.1. Subunits of complexes III and IV are down-regulated

The whole cell lysates from non-induced and induced cells were analyzed by Western blot using a panel of specific polyclonal antibodies, including those directed against MRP1 and MRP2 proteins. Both proteins were shown to be efficiently eliminated in their respective knock-downs 6 days post-induction (Fig. 1A). Since no specific antibodies against mitochondrial-encoded subunits of the respiratory complexes of *T. brucei* are available, we could determine only the presence of selected nuclear-encoded subunits. Western analysis of trCOIV, cox6 and apoC showed a marked decrease in their abundance by day 4, with the proteins being almost undetectable by day 6 after RNAi induction. However, the subunit b of F_0F_1-ATP synthase (complex V) against which antibody is available was not decreased in any of the RNAi-interfered cell lines. This observation was not surprising, as the depletion of the MRP proteins does not affect the pan-editing of subunit A6 of complex V [4]. The levels of the nuclear-encoded TAO localized in the inner membrane and mitochondrial heat-shock protein (hsp) 70 remained unaltered in the analyzed cells, the latter protein being used as a control (Fig. 1B).

3.2. Complexes III and IV are destabilized

The observed down-regulation of subunits of complexes III and IV (trCOIV, cox6 and apoC) prompted us to analyze the entire complexes in the purified mitochondrial vesicles solubilized with mild detergents and separated by 2D blue native/Tricine–SDS-PAGE electrophoresis. The complexes were well-separated using 2–15% gradient gel in the first dimension, and their position was identified by the mobility of stainable subunits and by the comparison with gels obtained from *T. brucei* under similar conditions [10,14,15].

At day 6 after RNAi induction, when the MRP proteins are virtually eliminated in the studied cell lines and the growth defect just became apparent [4], a significant decrease of complexes III and IV followed. In the MRP1 knock-down, complex IV was undetectable, while traces of complex III were still present (Fig. 2). The effect on respiratory complexes was even more pronounced in the MRP2 and MRP1+2 RNAi cells, in which both complexes were degraded. Furthermore, the double knock-down exhibited a decreased abundance of complex V (Fig. 2).

3.3. Activities of respiratory complexes are altered

Another approach to assay for the respiratory complexes in the MRP RNAi cells was to measure specific activities of these complexes. The activity in mitochondrial lysate of cytochrome *c* reductase (complex III) was established in a buffer containing reduced decylubiquinone via a change of absorbance of cytochrome *c* as described elsewhere [10]. In three independent RNAi induction experiments in which MRP1, MRP2 or both have been targeted, complex III activity was reduced to ∼19, 28 and 20% of that of the untreated cells, respectively (Table 1).

The assay for cytochrome *c* oxidase (complex IV) requires, due to an activity lower by two levels of magnitude when compared to complex III, high number of cells. Three measurements of independently interfered MRP1, MRP2 and MRP1+2 cells revealed a decrease to ∼20, 24 and 7.5% of the values in the non-induced cells, respectively. The activity of NADH dehydrogenases remained virtually unchanged in all three cell lines as measured in the respective mitochondrial lysates (Table 1).

We have further employed an alternative approach of measuring activities of the respiratory complexes. By direct staining in the gel after blue native electrophoresis, the activity of complex IV can be visualized as a colored precipitate [10]. *L. tarentolae* and *Phytomonas serpens*, two kinetoplastid species in which activity of this complex is strongly pronounced and absent, respectively, have been used as positive and negative controls. While some trace activity of complex IV was visible in the induced MRP1 knock-downs (Fig. 3A; MRP1(+)), the same

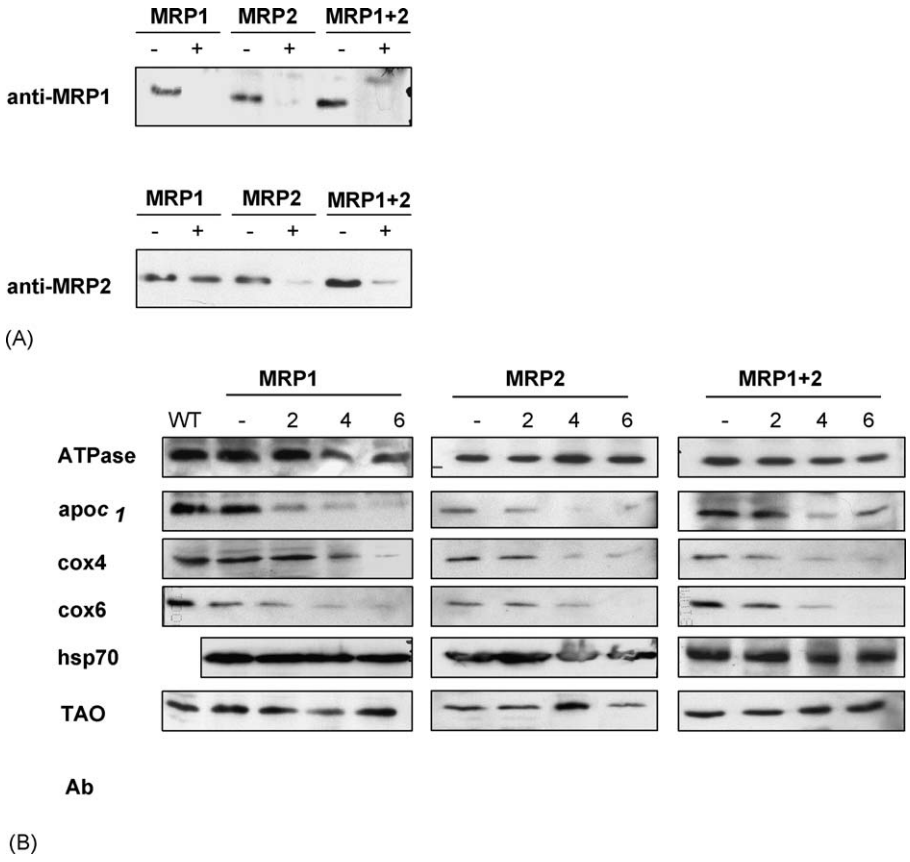

Fig. 1. Effect of down-regulation of MRPs on protein levels. Protein levels were analyzed by Western blot analysis in extracts from single and double knockdown cell lines in the parental strain 29-13 (WT), before RNAi induction (−), and 2, 4 and 6 days of RNAi induction. Each lane was loaded with protein from ∼10^7 cells and blots were immunostained using specific polyclonal antibodies. (A) Western blot analysis of MRP1 and MRP2 in non-induced cells (−) and those 6 days of RNAi induction (+). (B) Western blot analysis of ATP synthase subunit b (ATPase), apocytochrome *c* (apoC), cytochrome *c* oxidase subunit (cox4 and cox6), and trypanosome alternative oxidase (TAO). Mitochondrial anti-hsp70 antibody was used as a loading control.

activity appeared to be totally eliminated in the induced MRP2 and MRP1+2 cells (Fig. 3A; MRP2(+) and MRP1+2(+)). The colored precipitate was quantified, revealing a reduction of activity to 16, 8 and 0.5% of the non-induced values, respectively.

We have also attempted to detect the complex I activity, the presence of which in *T. brucei* procyclics was confirmed only very recently [10]. First detection of this complex in *L. tarentolae* indicates that in this trypanosomatid complex I is larger than in *T. brucei*, where its estimated size is ∼650 kDa (Fig. 3B). About half of the complex I activity was eliminated in cells interfered against MRP1 or MRP2 (Fig. 3B). However, in the double knock-down, histochemical detection failed to detect any complex I activity (Fig. 3B; MRP1+2(+)). Quantification of the colored precipitates showed their repression in the induced MRP1, MRP2 and MRP1+2 cells by 48, 50 and 96%, respectively.

Table 1
Activities of respiratory complexes

Type of cells	Induction	NADH dehydrogenase activity (μU mg^{-1})	%	Cytochrome *c* reductase activity (mU mg^{-1})	%	Cytochrome *c* oxidase activity (mU mg^{-1})	%
L.tarentolae		183 ± 36		678 ± 259		5.66 ± 0.19	
P. serpens		81 ± 28		19 ± 8		0.155 ± 0.05	
T. brucei parental strain		61 ± 6		511 ± 53		1.64 ± 0.43	
MRP1	Non-induced	92 ± 34	100	214 ± 36	100	2.11 ± 0.19	100
MRP1	Induced	115 ± 50	125	41 ± 19	19	0.43 ± 0.013	20
MRP2	Non-induced	42 ± 17	100	282 ± 32	100	0.85 ± 0.36	100
MRP2	Induced	37 ± 2	88	81 ± 23	28	0.2 ± 0.06	24
MRP1+2	Non-induced	45 ± 11	100	320 ± 67	100	1.6 ± 0.31	100
MRP1+2	Induced	45 ± 28	100	66 ± 15	20	0.12 ± 0.04	7.5

Activities of complexes I, III and IV were measured in mitochondrial lysates as described in Section 2. Percentage of activity is shown in cells 6 days of RNAi induction with the activity of a given non-induced RNAi cell line set at 100%.

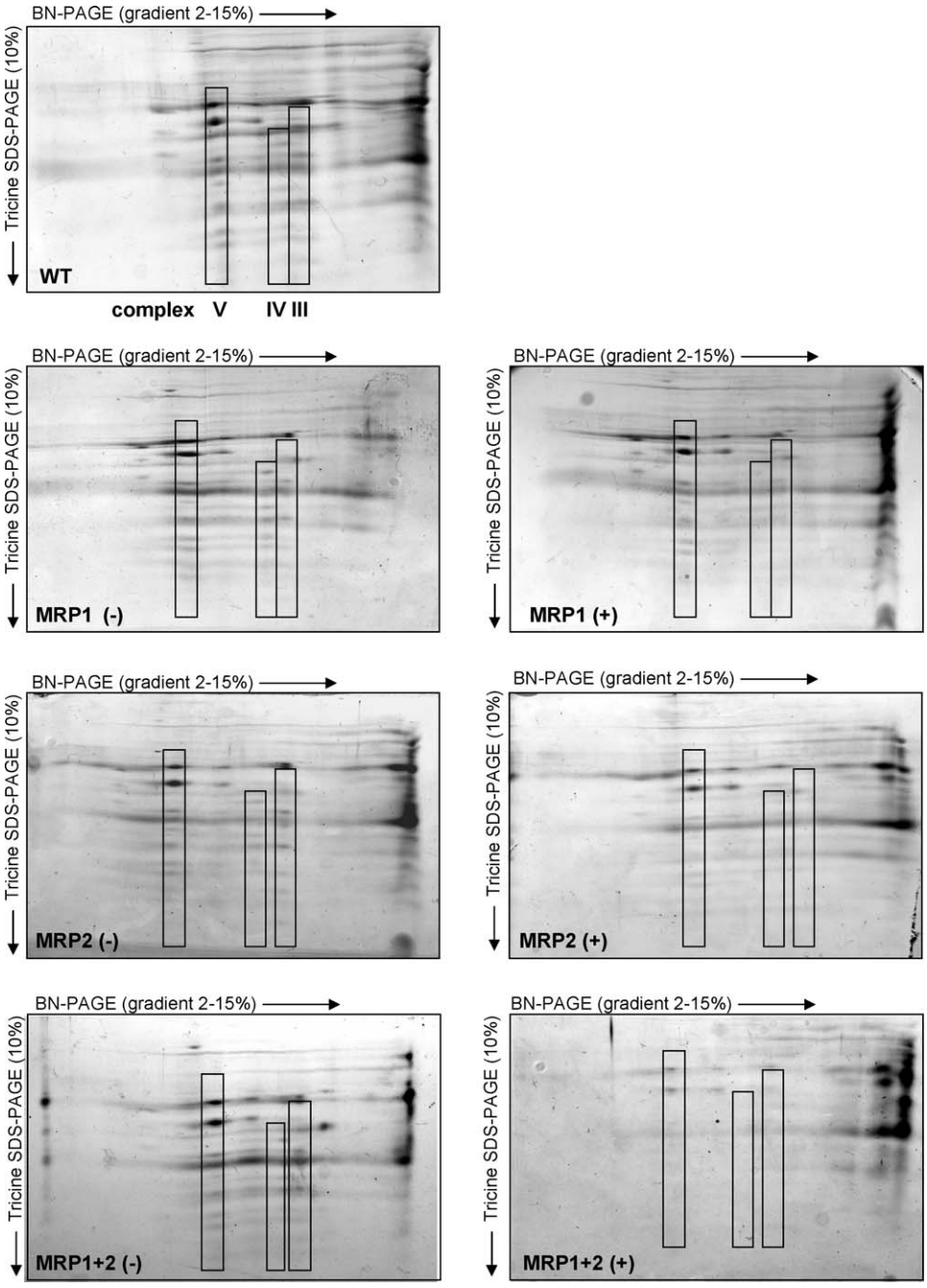

Fig. 2. 2D gel analysis of mitochondrial membrane proteins. Respiratory complexes in mitochondrial lysates from non-induced (left panel) and induced cells (right panel) were resolved in 2D 2–15% gradient blue native/10% Tricine–SDS-PAGE gels and stained with Coomassie Brilliant Blue. Positions of the respiratory complexes III, IV and V are indicated by boxes in the gel.

3.4. Switch to alternative oxidase is associated with lower membrane potential

The disruption of RNA editing and/or increased destabilization of mitochondrial transcripts should influence the activity of all respiratory complexes that contain the mitochondrial-encoded subunits, whereas the activity of TAO encoded by the nuclear genome should not be altered. Western analysis of the non-induced and induced cell lysates with the anti-TAO antibody confirmed this presumption (Fig. 1B), but could not reveal the actual activity of this terminal oxidase. Therefore, we have monitored oxygen consumption in the MRP knock-downs using selective inhibitors that allowed us to distinguish between the two respiratory pathways of trypanosomatids: the cytochrome-mediated (KCN-sensitive) one, typical for all eukaryotes, and the unique alternative (SHAM-sensitive) pathway.

At day 6 post-induction the rate of oxygen consumption decreased by about 20% in the three knock-downs, mainly as a result of selective decrease in the cyanide-sensitive respiration. Upon the addition of KCN (final concentration 0.1 mM) to the

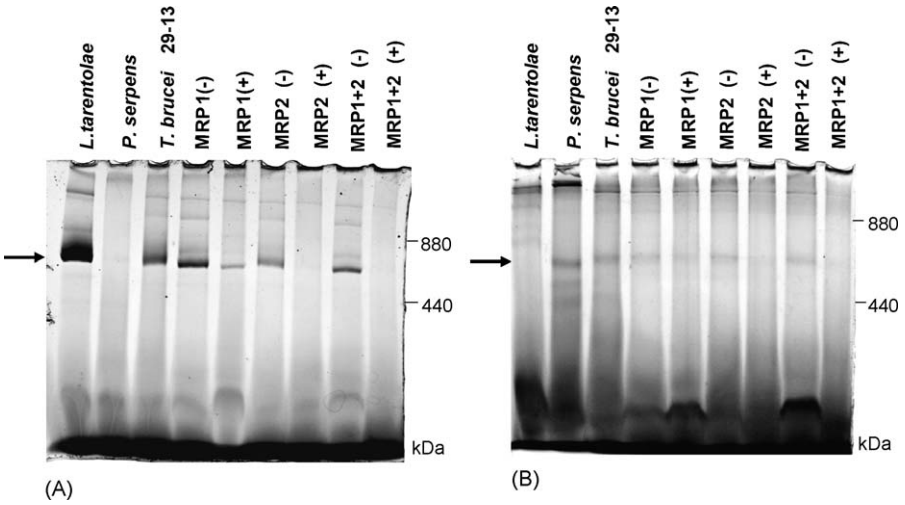

Fig. 3. Histochemical staining of respiratory complexes. Complexes IV (left panel) and I (right panel) in the mitochondrial lysates of *Leishmania tarentolae*, *Phytomonas serpens*, parental strain 29-13 (WT) of *T. brucei*, non-induced and induced cells 6 days of RNAi. Both complexes have been detected in 2–15% gradient blue native gels.

non-induced MRP cells, the rate of oxygen uptake dropped by about 40%, due to specific inhibition of complex IV by this drug. Subsequent addition of SHAM (final concentration 0.03 mM), a specific inhibitor of TAO, terminated oxygen consumption of these cells, leading within a few minutes to their death (Fig. 4A; black line). The addition of KCN to the induced MRP cells had a very small influence (about 10% decrease) on oxygen uptake rate (Fig. 4A; grey line). The lack of any effect was most apparent in the induced double knock-down, in which respiration remained virtually unaltered after the addition of KCN. These results indicate that the cytochrome-mediated respiratory chain participates in the flow of electrons either marginally or not at all in the MRP single and double knock-downs, respectively, and that respiration in these cells depends primarily on TAO. Relative contribution of the SHAM- and KCN-sensitive pathways to the overall respiration is shown in Fig. 4B, revealing the compensatory effect of functional up-regulation of the alternative SHAM-sensitive pathway on oxygen consumption.

Since TAO does not contribute to the maintenance of mitochondrial membrane potential, partial redirection of the electron flow to this terminal oxidase should be accompanied by the decrease of membrane potential. Indeed, its quantification by the uptake of TMRE using flow cytometry showed that in all induced knock-downs membrane potential was significantly decreased (28–54% depending on the cell line) (Fig. 5).

4. Discussion

Functional analysis of proteins involved in RNA editing in *T. brucei* and *L. tarentolae* is progressing at a rapid pace. So far, most components of the 20S editosome have been studied in knock-down or knock-out cell lines [3] and many more analyses are under way. Yet with the single exception of the down-regulation of one of the RNA editing terminal uridylyl transferases [9], the studies have primarily focused on the direct function of the target protein(s) in editing or stability of mitochondrial RNAs. Much less is known about the function of the so-called accessory factors, proteins with RNA-binding activity that are not permanent components of the 20S editosome [1–3,16]. While some of them have so far been implicated only with the metabolism of edited transcripts, both MRP proteins appear to have a more intricate role. Their ablation has a complex effect, strongly influencing only a subset of both never-edited and edited mRNAs [4,17]. A similar phenotype has been described for another accessory factor, RBP16 [18,19]. In order to appreciate the impact of the depletion of the abundant MRP proteins on the organelle, we decided to assay for general mitochondrial functions. Such an extended analysis may also provide novel insight into the (in)dispensability of subunits translated from the edited mRNAs on the respiratory complexes.

As mentioned above, the MRP proteins are essential for editing and/or stability of subunits of complex I (down-regulation of never-edited ND4 and ND5; up-regulation of edited ND7), complex III (down-regulation of edited apoB) and complex IV (down-regulation of never-edited cox1). Moreover, lower efficiency of mitochondrial translation is anticipated, since the only mitochondrial-encoded and edited ribosomal subunit RPS12 is decreased in the MRP knock-downs by about 50% [4]. Only recently, it was experimentally proven that both edited and never-edited mRNAs are genuine templates for the synthesis of respective proteins [12,20,21]. The massive impact of the MRP depletion on editing and stability of edited and never-edited mRNAs is reflected in protein synthesis, as shown by lack of pulse-labeling of de novo synthesis of the apoB and cox1 proteins in the MRP knock-downs (M. Neboháčová, pers. commun.).

Antibodies directed against the nuclear-encoded subunits of complexes III and IV revealed their elimination in the absence of the MRP proteins. Such an effect indicates that the lower availability of mitochondrial cox1 and apoB caused the destabilization and/or higher turnover of the imported proteins. Indeed, direct analysis of the respiratory complexes using 2D blue native gel electrophoresis confirmed the ablation of complexes III and IV in the interfered cells. On the other hand, silencing of MRPs

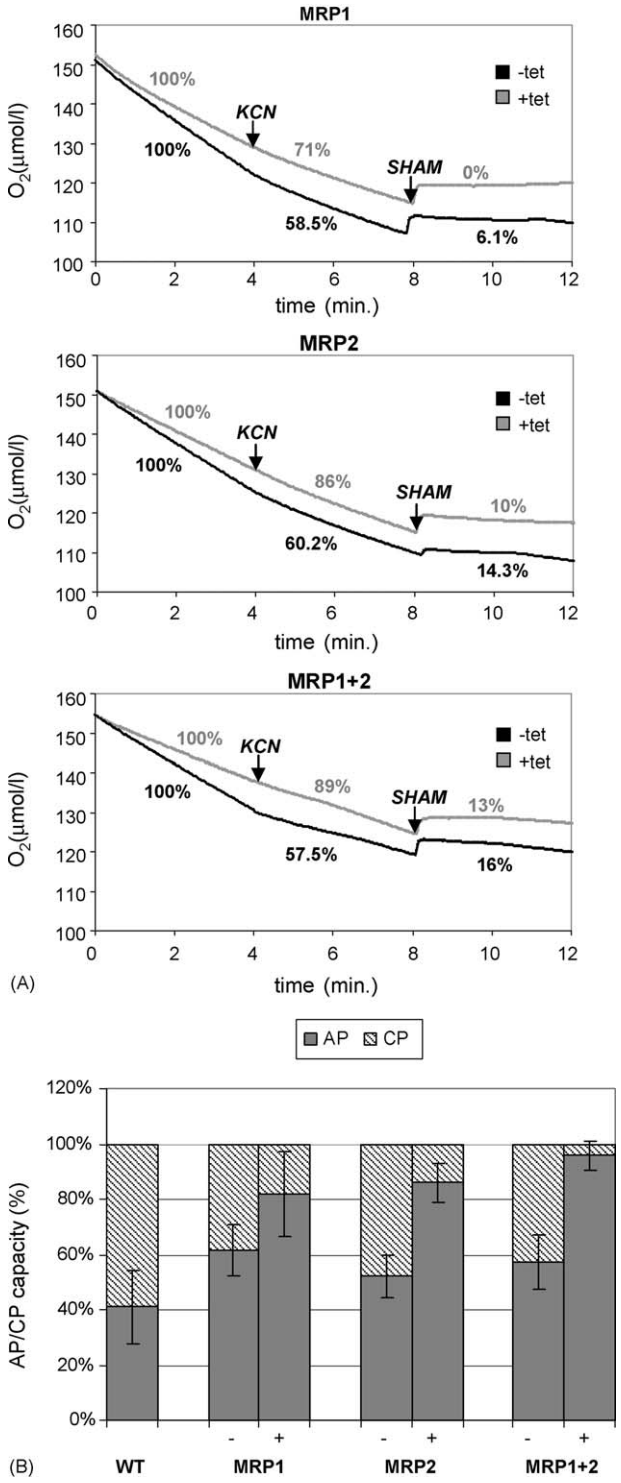

Fig. 4. Measurement of respiration. (A) The effect of down-regulation of MRP proteins on oxygen consumption of non-induced (black line) and induced cells (grey line) as monitored with an oxygen electrode. KCN (0.1 mM) and SHAM (0.03 mM) were added to cells incubated in SDM-79 medium at 27 °C. *x*-axis = time in min; *y*-axis = oxygen consumption in µmol O_2/l. The numbers next to the lines indicate percentage of (residual) oxygen consumption with respiration of non-induced and induced cells in the absence of both drugs set at 100%. (B) Relative contribution of alternative pathway (AP) and cytochrome-mediated pathway (CP) in parental strain 29-13 (WT), non-induced (−) and induced cells 6 days of RNAi (+). The amount of O_2 consumption inhibited by KCN was measured as the CP capacity. The mean and the S.D. values of three experiments are shown.

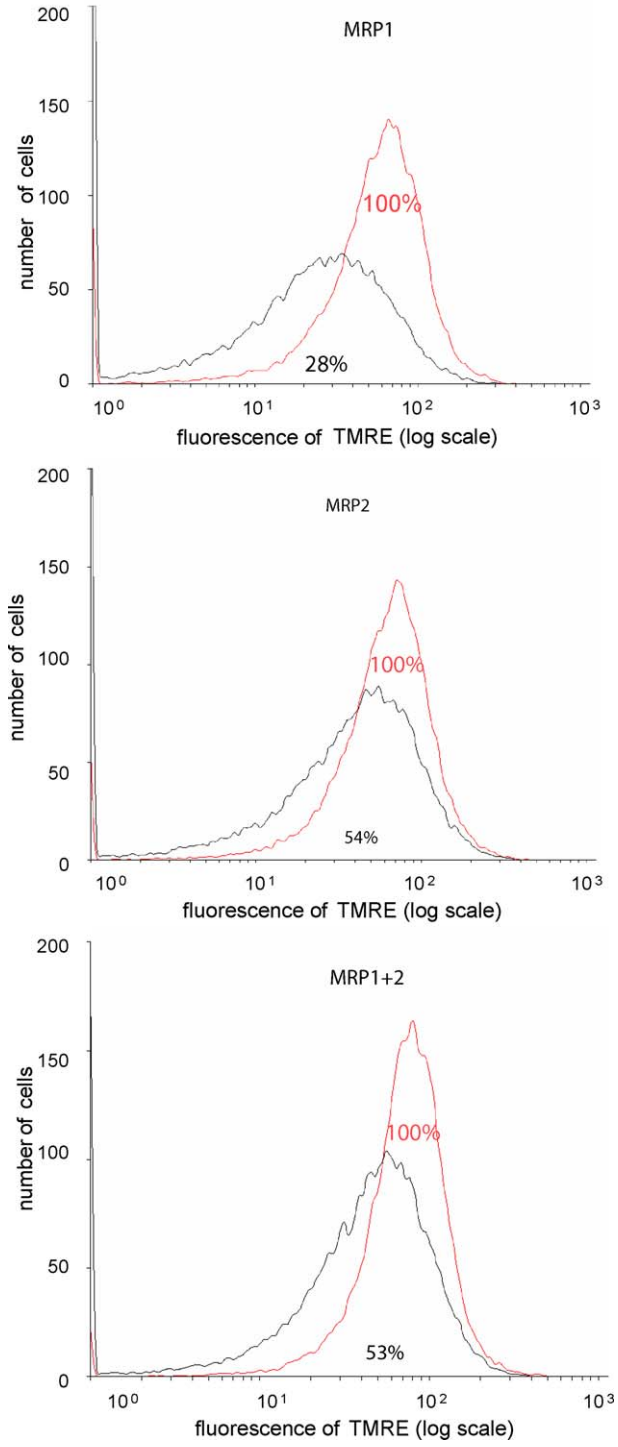

Fig. 5. Measurement of membrane potential. Mitochondrial membrane potential was measured in non-induced cells (red line) and in cells 6 days of RNAi induction (grey line) that were, after the incubation with 0.4% tetramethylrodamine ethyl ester for 20 min, analyzed using flow cytometry. The fluorescence distribution was plotted as a frequency histogram.

had no effect on the abundance of the nuclear-encoded subunit b (p18) (this work) and mitochondrial-encoded ATP synthase subunit 6 [4]. Complex V remained at or close to the wild type level in the MRP1 and MRP2 knock-downs, however, its assembly was affected in the MRP1+2 knock-down.

This is in a good agreement with other systems, where rapid degradation of proteins not incorporated into the respiratory complexes has been observed. In yeast with defective mitochondrial protein synthesis, nuclear-encoded subunits of complexes III and IV continue to be synthesized and imported into the organelle, but assembly of these complexes is impaired [22,23]. Similar observation has been made in mammalian cells with the mitochondrial protein synthesis blocked by ethidium bromide. The synthesis of nuclear-encoded subunits of complexes I, III and IV was not affected but, upon import, the proteins were rapidly degraded [24].

Currently, no antibodies against components of complex I are available and, in fact, its very existence in *T. brucei* remains under debate. However, the presence of putative homologues in the mitochondrial [25] and nuclear genomes of *T. brucei*, *T. cruzi* and *L. major* (Z. Verner, A. Horváth, J. Lukeš, unpubl. results), in combination with biochemical evidence [10,11] speak strongly in favor of an active participation of complex I in the oxidative phosphorylation of kinetoplastid flagellates. For the detection of this complex in analyzed cell lines, we have used a newly developed histochemical method [10]. In the absence of a single MRP protein, about half of the activity of complex I was retained. However, since in the MRP1+2 knock-down both ND4 and ND5 mRNAs were strongly decreased [4], the respective complex was down-regulated to an almost undetectable activity. This result shows a direct dependence of the function of complex I on the availability of mitochondrial-encoded subunits, which in other eukaryotes also represent key subunits of this complex [26]. However, the measurement of the NADH dehydrogenase activity revealed no changes, which can be explained by the anticipated presence of alternative dehydrogenase(s) in the mitochondria of *T. brucei* cells [27].

The activity measurement, histochemical detection and 2D native gel electrophoresis confirmed the destabilization of complex IV. The effect was weaker in the MRP1 RNAi cells, which is in a good agreement with only moderately decreased abundance of the respective mitochondrial transcripts in this cell line. All in all, our data show that mitochondrial subunits of complexes I, III and IV are absolutely essential for the function of these complexes in *T. brucei*.

Since the MRP1-silenced cells showed only a barely noticable growth phenotype and generally less pronounced effect on mitochondrial transcripts [4], these cells apparently retain a fraction of active respiratory complexes, whereas the down-regulation of MRP2 and MRP1+2 caused not only a strong disruption of mRNA editing and stability, but also the loss of respiratory complexes and cessation of growth. Although the current view holds that in the presence of glucose, oxidative phosphorylation is not the main route of ATP generation in procyclic *T. brucei* [28–30], the extent of down-regulation of the respiratory complexes seems to be in direct correlation with the viability of the interfered cells. It follows that in the absence of this pathway, less bulk ATP is produced in the organelle since TAO does not produce ATP and merely serves as an effector terminal oxidase.

The capacity of procyclics to shift from the cytochrome-mediated pathway to an alternative one was demonstrated using specific inhibitors of the respiratory complexes III and IV [31], by knocking-down nuclear-encoded subunits of these complexes [10] and terminal uridylyl transferase indispensable for editing of their mitochondrial-encoded subunits [9,32], and finally by overexpressing TAO [33]. In this study we see the same effect, supplemented with the additional disruption of complexes I and V. The activity of TAO is increased in the induced cells, without the increased abundance of the protein, which can be explained by the predicted overcapacity of TAO in *T. brucei* [34]. Since TAO does not contribute to the mitochondrial membrane potential, the low potential observed in the MRP knock-downs can either be sustained by the residual activity of complex I or rather by the activity of complex V. When compared with the other respiratory complexes, the latter complex seems to be least sensitive to the inhibition of mitochondrial protein synthesis [24]. Moreover, ATP synthase can generate proton gradient in the bloodstream stages by hydrolyzing ATP [35–37], and it is possible that it may switch to this activity in the genetically modified procyclics, and up-hold the potential, albeit at lower levels. However, the hypothesis regarding the possible role of ATP synthase in the generation of proton gradient in the mutant cell lines remains debatable and requires further investigation.

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